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MICRO-ORGANISMS AND DISEASE.

MICRO-ORGANISMS AND DISEASE

AN

INTRODUCTION INTO THE STUDY OF SPECIFIC MICRO-ORGANISMS.

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BY

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THIS BOOK

Is Respectfully Dedicated

BY

THE AUTHOR.

PREFACE.

THE following book, with few additions, is a reprint of a series of articles that have appeared in the *Practitioner* (1884). Not pretending to be in any sense an exhaustive treatise it will, I hope, nevertheless serve to illustrate most of the important points bearing on investigations into the life history of micro-organisms connected with infectious diseases.

I must not omit to mention that most of the investigations recorded here were carried out for the Medical Department of the Local Government Board during the past ten years.

E. KLEIN.

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MICRO-ORGANISMS AND DISEASE.

INTRODUCTION.

THE relation of micro-organisms to the infectious diseases is admitted to be very intimate; and although it may not be quite so universal as some are inclined to assume, it is nevertheless definitely proved to exist as regards some of the infectious maladies affecting man and brutes. In order to pass in review all the ascertained facts and observations in this vast and constantly-growing field of pathology, and to appreciate and to assign their true value to the many observations bearing on this relation of micro-organisms to disease, it is necessary that the reader, and still more the worker in this field, should be enabled to criticise the observations and facts brought forward by the numerous writers on the subject, for otherwise he would probably take as proved what has really not passed beyond the stage of possibility. And it is this point which requires the most careful attention, viz., to be able to see at a glance that, owing to the imperfect or faulty method of investigation employed, or that, owing to certain inferences incompatible with the general laws and general tendency of the well-founded and experimentally proved facts, the statements set forth in a particular observation or series of observations are not to be accepted.

In all investigations of the relation of micro-organisms to disease it is necessary to bear in mind that, as Koch[1] has pointed out, no observation can be said to be complete, or, one should rather say, in no instance can it be said to have been *satisfactorily proved*, that a particular infectious disease is due

[1] *Die Milzbrand-impfung*, Cassel and Berlin, 1883.

to a particular micro-organism if any one of the following conditions remains unfulfilled :—(1) It is absolutely necessary that the micro-organism in question is present either in the blood or the diseased tissues of man or of an animal suffering or dead from the disease. In this respect great differences exist, for in some infectious diseases the micro-organisms, although present in the diseased tissues, are not present in the blood ; while in others they are present in large numbers in the blood only or in the lymphatics only. These points will be considered hereafter in the special cases. (2) It is necessary to take these micro-organisms from their nidus, from the blood or the tissues as the case may be, to cultivate them artificially in suitable media, *i.e.* outside the animal body, but by such methods as to exclude the accidental introduction into these media of other micro-organisms ; to go on cultivating them from one cultivation to another for several successive generations, in order to obtain them free of every kind of matter derived from the animal body from which they have been taken in the first instance. (3) After having thus cultivated the micro-organisms for several successive generations it is necessary to re-introduce them into the body of a healthy animal susceptible to the disease, and in this way to show that this animal becomes affected with the same disease as the one from which the organisms were originally derived. (4) And, finally, it is necessary that in this so affected new animal the same micro-organisms should again be found. A particular micro-organism may probably be the cause of a particular disease, but that really and unmistakably it is so can only be inferred with certainty when every one of these desiderata has been satisfied.

It will be my aim in the following pages, first to describe the methods that may be employed with success in investigations bearing on the relation of micro-organisms to disease; secondly, to describe in conformity with reliable observations the morphology and physiology of the micro-organisms that bear any relation to disease ; and thirdly, to enumerate the observations that have been made in recent years to prove the existence of such an intimate relation. Last, but not least, we shall consider the precise relation of the particular micro-organisms to the causation of disease.

CHAPTER I.

MICROSCOPIC EXAMINATION.

For the examination of micro-organisms good high powers are essential, at the least a power magnifying 300 to 400 linear diameters. Zeiss' D or E and Zeiss' or Powell and Lealand's oil immersion 1-12th or 1-16th inch will be found sufficient for all purposes. In the case of tissues stained with aniline dyes a good substage-condenser such as Abbé's or Powell and Lealand's, is invaluable. I use Zeiss' stand with Abbé's condenser, open diaphragm, and plane mirror. As Koch [1] pointed out, and what is now universally acted upon, stained specimens mounted in Canada-balsam solution or Dammar varnish, when examined on an Abbé's condenser, show the micro-organisms with extreme clearness and sharpness.

The examination of the morphological characters of an organism is carried out on fresh unstained, as well as on fresh stained microscopic specimens. Although the latter method is, for reasons hereafter to be mentioned, by far the most perfect and reliable one, it is nevertheless important to ascertain as far as possible the appearances, chemical reactions, and general morphology of perfectly fresh specimens. Blood, juices, tissues, and fluids in which the micro-organisms have been growing, are subjected directly, without any previous preparation, to microscopic examination. With artificial nourishing media in which micro-organisms have been growing, the examination of fresh specimens is of great importance, for the reason that the organisms can be easily identified and their size and general morphological characters be more correctly ascertained than after drying, hardening, and staining. Besides, the chemical reactions can be satisfactorily studied in fresh

[1] *Die Aetiologie d. Wundinfectionskrankheiten*, p. 84, Leipzig, 1879. Translated as *Traumatic Infective Diseases* (New Syd. Soc.), London, 1880.

specimens only. All one has to do is to draw up with a capillary pipette or to take up with the point of a needle a drop or particle of the material, to place it on an object-glass, and to cover it up with a thin cover-glass. Where one has to deal with liquids, such as artificial nourishing fluids, blood, serum, tissue-juices, secretions, transudations, and exudations, no addition is required. In the case of more solid material, such as solid artificial nourishing material, bits of tissue, &c., the addition of a drop of neutral previously well-boiled saline solution (of 0·6 to 0·75 per cent.) is advantageous although not absolutely necessary, since by pressing down the cover-glass a layer of the material sufficiently thin for examination can be obtained. In some instances a bit of tissue can be teased out into fine particles by means of two clean needles. Where it is a question of micro-organisms sufficiently conspicuous by their shape, size, and general appearance, their identification in the fresh condition is not difficult; this is the case with bacilli, actinomyces, and mycelia, but in the case of micrococci, especially when isolated or in couples, and lying in blood, juices, or tissues, their recognition is often extremely difficult. When in large clumps, such as larger or smaller masses of zoogloea, or when in the shape of chains, the identification is not difficult; but in the more isolated state they are not easily recognised owing, as a rule, to the presence of granules or particles of various kinds, from which morphologically their distinction is well-nigh impossible. In such cases there are certain rules of thumb, if I may say so, which assist, although they do not absolutely insure, the diagnosis. These are the micro-chemical reactions. The addition of liquor potassæ leaves micro-organisms quite unaltered, whereas fatty and most albuminous granules alter or altogether disappear by it. Acetic acid from 5 to 10 per cent. strong does not affect micro-organisms, but albuminous and other granules become in most instances altered. These two re-agents, I think, are as reliable as any others; if they fail, then others like alcohol, chloroform, sulphuric ether, &c., are not of any greater help; but the latter re-agents may be used, for instance, when it is a question between fat-granules and micrococci, or crystals and bacilli.

Micro-organisms have a great affinity for certain dyes, especially aniline dyes, and therefore these are used with great success to demonstrate their presence, and to differentiate in many instances morphological details which in the unstained condition are not discernible. The staining is effected on fresh unaltered organisms, or after they have been dried. In the

first instance the process is carried out thus :—A microscopic specimen is made, and to it is added afterwards drop after drop of the dye, passing it through the specimen in the usual way of applying fluids to a microscopic specimen, *i.e* by adding with a capillary pipette the dye at one margin of the cover-glass and sucking it up with a strip of filter-paper applied to the opposite margin of the cover-glass. When the staining has taken place the excess of the dye is washed away with salt solution, water, or alcohol, or both, as the case may be (see below). Unless the organisms are embedded in continuous masses of solids, this method gives good results. In the latter case, say if they are embedded in a microscopic lump of tissue, or in a particular spot of a fine section of a fresh tissue, it is necessary, after having placed the lump or section on an object-glass, to drop the dye on to this previous to putting on the cover-glass. After some minutes the dye is allowed to run off by inclining the object-glass, and then the washing is proceeded with till all the excess of the dye is removed ; the mounting is then done by placing a drop of water or salt solution on the specimen and covering it with a cover-glass. In the case of sections through fresh and hardened tissues containing micro-organisms, the method of staining and of permanently mounting them as a whole is more complicated, and will be detailed presently.

When one has to deal with coherent masses of micro-organisms, present either in natural media (*i.e.* animal tissue), or artificial cultivations, such as zoogloea and pellicles of *micrococcus* or *bacterium*, these can be bodily transferred to a watch-glass, stained, washed, and mounted without much difficulty, either for immediate or permanent use. The permanent specimens are made in this way :—Place the sections or pellicle in a watch-glass containing the dye, leave it there till deeply tinted, take out with a needle or the like, wash in water, then in alcohol, leave here for five minutes or more till most of the excess of the colouring-matter is removed, then lift it on to an object-glass, spread well out, place on it a drop of clove-oil, and after a minute or two drain off the clove-oil, add a drop of Canada-balsam solution (in chloroform or benzol), and cover with a cover-glass. In some special instances such as the bacilli of leprosy and tuberculosis, double staining is required. With other organisms, such as the bacilli of glanders or tuberculosis, the washing is carried out, not with water but with acid (acetic acid and nitric acid respectively). All the details will be stated when dealing with these special organisms.

The method extensively and successively used for the demonstration and preservation of microscopic specimens of micro-organisms in fluids, as blood, pus, and juices, is that of Weigert and Koch, which consists in spreading out on a glass slide or cover-glass a very thin film—the thinner the better—of the fluid (artificial or natural culture medium), blood, pus, or juice, and drying it rapidly by holding it for ten to twenty seconds over the flame of a spirit-lamp or gas-burner. The most successful preparations are obtained when the heating is carried on for such a time that the film, having become opaque at first, rapidly turns transparent. Several drops of the aniline dye to be used are then poured over the specimen, and after remaining on it from five to thirty minutes or more are poured off. The aniline dyes used are those that are commonly known as having a great affinity for cell-nuclei (Hermann); they are also known as the neutral or basic, but not the acid ones. The most useful aniline dyes amongst them are those that are soluble in water; these are preferable to those soluble in alcohol, but in certain special instances (to be mentioned hereafter) some of them are of definite use. Methyl-blue, methyl-violet, vesuvin, Bismarck-brown, magenta, fuchsin, rosanilin, gentian-violet, Spiller's purple, eosin, dahlia, purpurin, iodine-green, are the aniline dyes commonly used. The washing is carried out in all cases, except with tubercle-bacilli and the bacilli of glanders, with distilled water, then with alcohol; this latter as a rule is to wash out the dye from all parts except the micro-organisms, and it is therefore necessary not to carry the washing further than this, but to carry it as far as practicable. After this, wash again with distilled water, dry, and mount in a drop of Canada-balsam solution. Throughout this process it is of course necessary always to remember on which side of the glass the specimen had been dried.

Double staining is carried out with any two of the above dyes; as a rule a brown and blue or violet, or a red and blue, are preferred. Some of the violet and purple dyes have the peculiarity that in some—not in all instances—they give to the preparation a double tint—some things appear blue, others more pink.

The process of double staining is carried out either for each dye separately—*i.e.* we first apply one dye, after some minutes wash in distilled water, and then apply the second dye—or the two dyes to be used are mixed and then used like a single dye.

In the case of tubercle-bacilli the staining is first done with a magenta mixture (Ehrlich's, Weigert's, or Gibbes' see

below), then washed for a few seconds with a 10 per cent. solution of nitric acid, then for a few minutes with distilled water. After this the preparation is stained with methyl-blue in the ordinary way. Or, after Koch's method, the specimen is first stained in alkaline methyl-blue (mixed with a 10 per cent. solution of caustic potash) for twenty-four hours, or for half an hour to one hour at a temperature of 40° C., and then stained in a concentrated solution of vesuvin. Wash it next with water, then with alcohol, dry, and mount in Canada-balsam solution.

In leprosy, the specimen on the glass is stained with magenta, then washed in distilled water, then stained with methyl-blue, washed, and mounted. With such organisms—as *e.g.* the micrococcus in the sputum of acute croupous pneumonia, and the micrococcus in gonorrhœal discharge—the staining is best carried out with a mixture of methyl-blue and vesuvin.

Weigert's double stain is very excellent for many purposes; it is prepared thus:—

Saturated watery solution of aniline, 100 ccm.

[This is made thus:—Aniline oil, 1 part, dist. water, 3 parts. Shake every half hour for four hours, and decant the water as the oil settles to the bottom.]

Saturated alcoholic solution of fuchsin, 11 ccm. Mix.

The sections are well stained in this mixture, then washed in distilled water; after this they are immersed for a few seconds in alcohol, and then transferred for one, two, or three hours to the following solution [Watson Cheyne, *Practitioner*, April 1883, p. 258]:—

Distilled water, 100 ccm.
Saturated alcoholic solution of methyl-blue, 20 ccm.
Formic acid, 10 minims.

After this, wash in alcohol, pass through clove-oil, and mount in Canada-balsam solution.

In examining fresh or hardened tissues for micro-organisms it is necessary to make thin sections, which can be easily done with the aid of any of the microtomes in common use, amongst which Williams's microtome, and especially Dr. Roy's ether-spray freezing microtome, are no doubt the best and easiest to manipulate. As regards hardened material, it is necessary to remember that the hardening must be carried

out properly, small bits—about a half to one cubic inch—of tissue being placed in alcohol, or better in Müller's fluid, and kept there; in the first instance, for two to five days; in the second, for from one to three weeks or more. Then small bits are cut out, of which it is desired to make sections. Those hardened in spirit must be soaked well in water to enable the material to freeze, then superficially dried with blotting-paper, and then used for cutting sections with Roy's microtome. Those hardened in Müller's fluid are at once superficially dried with blotting-paper and cut. When making sections with Williams's freezing microtome it is necessary to soak the material first in gum mucilage and then to freeze and to cut. Fresh tissues are at once cut with the freezing microtome, the sections placed in 0·6 per cent. of saline solution, floated out and well spread out, and then stained by transferring them in this condition, *i.e.* well spread out, into a watch-glass containing the dye. The sections of hardened tissues are floated out in water, well spread out, and then transferred to the dye or dyes as the case may be.

It is necessary to prevent too much shrinking of the sections, especially those of fresh tissues; for this reason it is advisable to float the sections in the saline solution or water, as the case may be, on a broad lifter or spatula, to spread them well out upon it, and to transfer them carefully into the dye, then into the dish with water used for washing off the excess of the dye, to transfer them, well spread out on the lifter, to alcohol, then after several minutes to oil of cloves, and finally on to a glass slide, on which they are mounted in the usual manner with Canada-balsam solution, the excess of clove-oil being previously drained off.

It is advisable, although not absolutely essential, to keep the sections in a well spread-out condition for a few seconds in alcohol before placing them into the dye.

CHAPTER II.

PREPARATION OF CULTURE MATERIAL.

ARTIFICIAL cultivations of micro-organisms in suitable nourishing media in the incubator (Fig. 1) at temperatures

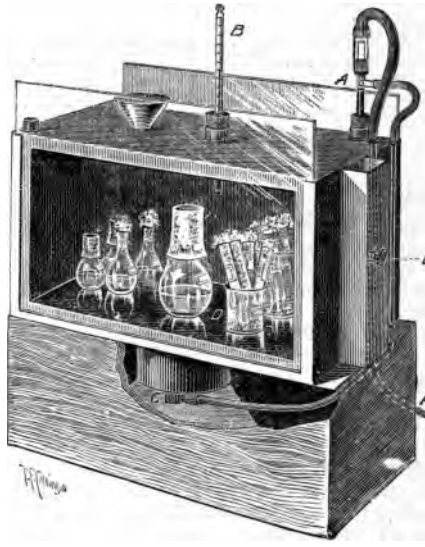


FIG. 1.—INCUBATOR, WITH PAGE'S REGULATOR.

A. Page's Regulator.—This consists of a tube filled with mercury, and immersed in the water surrounding the chamber of the incubator. In the upper part of the tube, above the mercurial column, is a fine open glass tube, having

near the lower end a fine hole. When the temperature of the water rises, the mercurial column rises, and at a certain temperature rises above the lower open end of the small inner glass tube just mentioned. If this point is reached, then the burner at C receives only the amount of gas that passes through the fine lateral hole of that inner glass tube. If the temperature of the water falls, the mercury falls, and the lower end of the inner glass tube becomes again free, and now the burner at C receives a much greater supply of gas. If so, the temperature of the water again rises, the mercury rises, obstructs the lower end of the inner glass tube, the supply of gas is reduced to what can pass through the fine lateral hole, and consequently the temperature again falls, and so on. To adjust the regulator it is necessary when the thermometer indicates the required degree of temperature to push the outer large glass tube, and with it the inner tube, of the regulator so far down that the top of the mercurial column just obstructs the free end of the inner glass. The temperature then regulates itself for the reasons stated previously. These regulators are sufficient for all practical purposes when it is not a question of small differences in temperature, since they are tolerably constant within one or two centigrades. The trouble one experiences in the working of these and other similar regulators arises from the inconstancy of the main gas supply, this, as is well known, varying within wide limits. The stopcock, E, obviates this to a limited extent, when this is put at an angle of 45° only a limited amount of gas passes from the main supply tube to the regulator, and therefore the variations in pressure of the gas are not felt to their full extent. A Sugg's regulator interposed between E and the main supply tap is very useful.

B. Thermometer to indicate the temperature in the chamber.

C. Gas burner

D. Chamber of incubator.

E. Stopcock to regulate when required the supply of gas.

F. Main supply.—The upper, lower, right and left walls of the incubator are made of a double layer of tin; between the two is water. The front and back of the chamber are closed by a movable glass plate.

An excellent incubator for constant temperature is made by the Cambridge Scientific Instrument Company. It has a double gas supply: one small permanent flame, and a second one subject to the regulator.

varying between 30° and 40° C., are necessary in order to study more accurately the life-history of the septic as well as the pathogenic organisms. Moreover, large numbers of them become available in a short time, and their relation to disease can be tested more conveniently. For if it should be found that, having carried on outside the animal body successive cultivations of a particular organism, the re-introduction of this cultivated organism into the animal body is again productive of the same disorder as before, then the conclusion becomes inevitable that this organism is intimately related to the causation of the disease. It must be conceded that after several successive cultivations in fluids any hypothetical substance supposed to be the *materies morbi*, and introduced at first from the blood or tissues, being in a very diluted condition in the first cultivation, would after several cultivations be practically lost. But if this last cultivation should be found to act in the same manner pathogenically, *i.e.* if every droplet of it, charged with the new brood of the

organism, nevertheless possesses full pathogenic power, then it is logical to say that this pathogenic property rests with the organism. For this and other reasons it is of essential importance to be able to carry on successive cultivations of one and the same organism without any accidental contamination or admixture, *i.e.* it is necessary to carry on *pure cultivations*.

ARTIFICIAL CULTIVATION MEDIA.

A.—Fluids.

As fluid nourishing material the following are used with preference :—

1. *Broth made from meat—pork, beef, rabbit, chicken.*—The connective tissue and fat are first cut out from the fresh meat —in the case of rabbit or chicken the whole animal without head or viscera is used—and then placed in water and boiled. Generally for each pound half an hour's good boiling is allowed. With regard to the quantity of water, each pound of meat ought to yield ultimately at least one pint of broth. When boiled, the broth is allowed to stand, the fat is skimmed off, and the broth well neutralised by adding liquor potassæ or, better still, carbonate of sodium.

The fresher the meat the less acid (sarcolactic acid) is in the broth before neutralisation. The broth is then filtered through a filter, previously overheated (see below), into flasks previously sterilised (see below). If the broth is not clear after once filtering it is filtered again. If not clear then, it is allowed to stand for several hours. A fine sediment is found at the bottom of the vessel, and from this the clear supernatant fluid is decanted into a sterilised vessel. The broth, if not clear after the first filtering, can be cleared by boiling it with the white of an egg. The now clear fluid is filtered again. The flasks which receive the broth are well plugged with sterilised cotton-wool (see below). In this state the flask is placed over a Bunsen burner (Fig. 2) on a wire netting and boiled for half an hour or more ; during the boiling the cotton-wool plug is lifted out for half its length. The flask ought not to contain more broth than about one-half or two-thirds of its volume, to prevent the broth from rising too much and wetting the plug. When turning off the flame the plug is pushed down so as fully to plug the neck and mouth of the flask ; a beaker with sterile cotton-wool cap is placed over

the mouth of the flask (Fig. 3), and this is allowed to stand for one night. Next day the boiling is repeated for half an hour or more in the same manner as before. If the meat has been fresh and the vessels and cotton-wool have been sterile, twice boiling is found sufficient to destroy every impurity. But to make sure, the broth is placed in the incubator and kept there for twenty-four hours at a temperature of 32°—38° C., and then boiled on the next day for half an hour in the usual way. The supposition is made, that if by any chance after twice boiling the broth it should contain unchanged spores of bacilli—the only organisms that will resist boiling, although

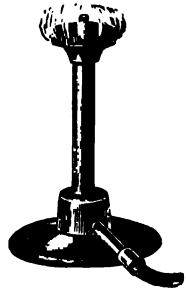


FIG. 2.—A BUNSEN BURNER, WITH ROSE FOR BOILING FLUIDS IN TEST TUBES.

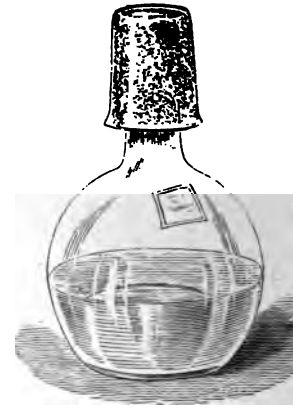


FIG 3.—A FLASK CONTAINING STERILE STOCK FLUID.

they do not resist boiling for more than half an hour—the spores would germinate into bacilli when kept for twenty-four hours in the incubator at 32°—38°, and these would then be killed by the third boiling. As a matter of fact I have not as a rule found any contaminating germs survive the second boiling. It is of course to be borne in mind that during the first as well as second and subsequent boiling the cotton-wool plug is not removed from the mouth of the flask, but is only raised out half its length from the neck. The cotton-wool and he cotton-wool cap and beaker are replaced immediately or

simultaneously with the turning off of the burner. This broth so prepared is placed in the incubator at 32°—38° C. and kept there from one to three weeks. If, as is generally the case, it remains limpid, it is considered completely sterile.

2. *Peptone and Sugar Solution.*—Beef peptone (Savory and Moore's) is dissolved in distilled water, over a burner, to the amount of about 2 per cent.; to the solution is added cane sugar to the amount of about 1 per cent.; so that every 100 ccm. of the fluid contains two grammes of peptone and one gramme of sugar. When dissolved it is well neutralised and then filtered (the vessels being of course also in this, as in all other cases, sterilised by overheating) into flasks, and treated in the same manner as the broth.

The same fluid can be used without the addition of the cane-sugar; or peptone dissolved to the amount of 1 to 2 per cent. in broth.

3. *Buchner's Fluid.*—10 parts of Liebig's extract, and 8 parts of peptone, in 1,000 parts of water.

4. *Hydrocele Fluid* (Koch).—A new or well sterilised (by over-heating) trocar and cannula are used for the tapping; to the cannula is fixed an india-rubber tube that has been soaking in strong carbolic acid solution for forty-eight hours. The distal end of the tube is introduced carefully and rapidly into the neck of a sterilised flask plugged with sterile cotton-wool, and the fluid thus allowed to flow into the flask to about two-thirds of its volume. This is then exposed in a water- or sand-bath to a temperature of from 58° to 62° C. for three to five hours on five or six consecutive days. Placed then into the incubator at 32°—38° C. for from one to three weeks, the fluid remains limpid.

5. *Blood Serum* (Koch).—A glass cannula and india-rubber tubing are soaked for forty-eight hours in strong carbolic acid; the cannula is tied into the carotid artery of a healthy sheep, and the arterial blood, after opening the clip at the proximal end of the artery, is allowed to flow into a sterile flask, the distal end having been introduced into the neck of the flask as above. After letting it stand for twelve to twenty-four hours the clear serum is taken off by means of a large sterilised glass pipette or glass syphon; this is carefully introduced between the cotton-wool plug and glass neck, and then discharged into a sterile plugged flask or large test-tube, the pipette or syphon being also here carefully introduced between cotton-wool plug and glass.

This stock serum is then heated for successive days in the same manner as the hydrocele fluid.

Of less common use are:

6. *Pasteur's Fluid.*—In 100 parts of distilled water are dissolved 10 parts of pure cane-sugar, 1 part of ammonium tartrate, and the ash of 1 part of yeast.

7. *Cohn's Fluid.*—100 ccm. of distilled water, 1 gramme of ammonium tartrate, no sugar, and instead of the ash of yeast are substituted (A. Mayer) 0·5 gramme of potassium phosphate, 0·5 gramme of crystallised magnesium sulphate, 0·05 gramme of (tribasic) calcium phosphate. These two fluids are sterilised in the same manner as the broth and peptone solutions. Pathogenic organisms do not thrive in either of these two fluids.

B.—Solids.

The solid media have the great advantage over the fluids that in the former artificial cultures can be carried out more easily; as, owing to the resistance the solid basis offers to the growth of the organisms, they remain more limited to the spot or spots on which they are sown, and therefore can be watched more easily; besides, an accidental contamination, i.e. a growth appearing at a spot at which no sowing was made, can be recognised at once. These advantages are perhaps of the greatest use when it is intended to grow the organisms on a surface exposed to the influence of air—of course protected from contamination with other organisms.

These advantages of solid media have been very minutely pointed out by Koch in his researches on pathogenic bacteria.[1]

As solid media are used:

1. *Slices of Boiled Potato or Boiled White of Egg or Paste* (Fokker, Schröter, Cohn, Wernich).—Although these are of great use in the study of hyphomycetes, and especially of pigment-bacteria, they are not generally used in the case of other bacteria and pathogenic organisms. The progress of the growth of a particular organism sown out at a particular spot or line on the surface of these substances can be easily watched with the unaided eye. These substances when quite fresh are placed on flat glass dishes; these are covered with a well-fitting bell-glass, the space being kept moist by a piece of moist blotting-paper placed in the dish.

2. *Gelatine* (Brefeld, Grawitz, Koch).—This is used advantageously as a mixture with broth, peptone, beef-extract, blood-serum, or hydrocele fluid. Koch, who introduced this mixture, used it for the cultivation of bacteria on solids, to be exposed

[1] *Mittheilungen d. k. Gesundheitsamtes*, i. 1881.

to the air; the proportion of gelatine in the mixture was 2 to 3 per cent. But this mixture, although solid at ordinary temperature, does not keep solid in the incubator, not even at 20° C. I have found that at least 7·5 per cent. of gelatine must be contained in the mixture to keep it solid at 20° to 25° C. Above this temperature not even 11 per cent. gelatine will keep solid.

The finest (gold label) gelatine, in thin tablets, is cut up in small strips; these are soaked in distilled water (1 in 6) overnight, and then dissolved over a water-bath, well neutralised with carbonate of sodium, and filtered hot. If not clear, it is boiled with white of egg, and passed hot through sterilised fine calico. Then this fluid is mixed with half its bulk of broth, peptone solution, or beef-extract solution, so that there is 1 part of gelatine in 9 parts of fluid, or 11⅛ per cent. of gelatine. This mixture is boiled repeatedly and treated like broth, as described above. The mixture can, when cast solid, be liquefied by melting it on the water-bath, can be easily decanted into sterilised plugged test-tubes (see below), and can then be used as a good solid nourishing material for the cultivation of organisms up to 25° C.

The above gelatine solution without admixture can be boiled once or twice, and thus made sterile and kept as a stock. This can be used as an addition to blood-serum or hydrocele fluid; the mixture must be sterilised in the same way as serum or hydrocele fluid alone, *i.e.* exposed for five to seven days to a temperature of 58° to 62° C. Of course, whatever the proportions are in which the two are mixed, the mixture does not keep solid above 25° C. But by exposing it for from several days to several weeks to the heat of the incubator, the mixture can by evaporation be rendered practically solid for higher temperatures also.

3. More satisfactory, because capable of remaining solid at any temperature, is solid serum of blood, solid hydrocele fluid (G. Makins), and Agar-Agar (Koch).

The first, *i.e.* the serum of blood, and the second, *i.e.* the hydroeele fluid, can be made solid by heating the above sterile serum or hydrocele fluid respectively (see page 13) *gradually* up to 68°—70° C. In the course of an hour or two the material becomes solid, losing slightly its limpidity, but is sufficiently transparent for all practical purposes. By heating it rapidly, or heating it above 70°, it becomes solid, granular, and opaque. Of course, once thus made solid it cannot be liquefied again, and therefore must be already contained in the vessels (test-tubes and small flasks) in which

the growth of organisms is to be carried on. Or blood serum and hydrocele fluid can be rendered solid by exposing the sterilised material (see above), in sterile plugged test-tubes, to a moderate heat—*e.g.* in the incubator at 32° to 38° C.—for several weeks. Through evaporation the material is rendered solid. Thus treated it retains its limpidity in a perfect manner.

Agar-Agar, or Japan isinglass, is very difficult to obtain,[1] it is sold in the shape of very thin, shrivelled, transparent lamellæ, or narrow bands. It is soaked overnight in salt water (one part of Agar-Agar to five or six of salt water), and then dissolved on the water-bath ; well neutralised with carbonate of sodium, filtered and mixed with a third of its bulk of broth, peptone, or beef-extract solution. I use now as a rule peptone solution, as described above. Well boiled on two or three successive days, each time for thirty minutes to an hour, in sterile flasks, a sterile material is obtained, which is quite transparent, and remains solid up to a temperature of 45°—50° C., *i.e.* a temperature much higher than is ever used for the cultivation of micro-organisms. It becomes liquid at higher temperatures, and in case of necessity can be again subjected to boiling. Before considering it as perfectly sterile it ought to be kept like all other materials for from several days to several weeks in the incubator at 32°—38° C. If quite limpid after this time it may safely be considered as sterile.

Amongst all the solid media, I have found this mixture of Agar-Agar and peptone sugar solution to be the best in many respects. It is beautifully limpid and solid, and an excellent nourishing material. Agar-Agar alone without the admixture of peptone is not satisfactory as a culture medium.

[1] Messrs. Christy and Co., of 155 Fenchurch Street, have succeeded in obtaining for me large quantities of this material from Paris. I understand from my friend Dr. R. Maddox that this substance is in reality what the French call *Gelose*.

CHAPTER III.

VESSELS AND INSTRUMENTS USED IN CULTIVATIONS.

All vessels (flasks, test-tubes, beakers, filters, calico), to be used are first thoroughly sterilised by overheating. In the case of flasks and test-tubes, this can be done by exposing them thoroughly in *all parts* to the open flame of a large Fletcher's burner; *while thoroughly heated* the mouth is plugged with a good long plug (1 to 2 inches) of sterile cotton-wool, this being pushed in by means of overheated forceps. The plug in all cases must not be loose, but also not too firm—an error in the latter direction being of course preferable to one in the former. The cotton-wool plug may, if long enough, be single; or, if short ones are used, double. Or the flasks and test-tubes are placed in an air-chamber (see Fig. 4) heated by a large Fletcher's burner for several hours, up to between 130° and 150° C. In the case of small flasks and test-tubes this process is of course much more convenient, since a large number can be heated simultaneously. Beakers and glass filters to be used merely for a temporary operation are placed over a wire net on a tripod and heated by the flame of a Bunsen's burner. In the case of test-tubes which are to receive cultivation-fluids, I generally expose them, after having been cleaned and dried, in the air-chamber for several hours (three to six) to a temperature of from 130°—150° C.: while hot they are taken out *seriatim*, plugged with the sterile cotton-wool, and replaced in the air-chamber, and heated again for several hours. All this, and other operations to be described below, may appear to some rather tedious and unnecessarily complicated, but it cannot be too strongly insisted on that in these matters one cannot be too scrupulous. A slight relaxation may, and occasionally is,

followed by disastrous consequences in the shape of accidental contamination, and consequent loss of material prepared at the cost of much labour and time. Long experience in these matters has taught me that, although in some instances less scrupulous care has not been followed by bad results, still I have had also many unpleasant failures owing to slight laxity in these matters.

FIG. 4.—HOT-AIR CHAMBER FOR STERILISING TEST-TUBES AND COTTON-WOOL.

An iron chamber with double wall, the inner chamber having separate folding doors. In the inner chamber are placed the test-tubes, glasses, &c., and the cotton-wool, the latter in a loose condition. Both sets of doors are closed, and the apparatus heated by a large Fletcher's burner. A thermometer passing from the inner chamber through the upper wall indicates the temperature of the chamber.

Several weeks' work may be annihilated by a single omission. Sometimes one is perhaps in a slight hurry, and does not think the want of an additional heating of the test-tube or cotton-wool or an additional boiling of the fluid will be followed by any bad consequences. But, alas, nature does not take into account our convenience, and failure is our reward. If in any kind of experiments "overdoing" is an error in the right direction, it is in these very experiments in the cultivation of micro-organisms.

The *cotton-wool* used for plugging flasks and test-tubes is prepared by pulling up loosely a quantity of good cotton-wool and exposing it in a loose state in the air-chamber to a temperature of 130°—150° C. *for several hours, for several successive days.* The cotton-wool ought to be just brown, *i.e.* just singed. Too much charring makes it very brittle, and it is then difficult to make of it a satisfactory plug. The plug used should not be too firm and not too loose: in the former case it is not easy to lift it up quickly, and in the latter it does not close sufficiently well. Cotton-wool that has been kept, say only for a day or two in the air-chamber for three or four hours is not absolutely sterile; nor is cotton-wool that has been kept in a compressed state in the air-chamber for any number of days. The central portions remain under these conditions quite white and are not sterile. No cotton-wool that is not just brown, *i.e.* just singed, is safe from risk of impurity. No cotton-wool steeped in absolute alcohol, strong carbolic acid, or any other disinfecting fluid, for ever so many days or weeks, can be absolutely relied on.

FIG. 5.—A CAPILLARY PIPETTE, DRAWN OUT INTO FINE POINTS: LENGTH ABOUT 12 TO 14 INCHES.

As stated above, a plug of sterile cotton-wool tolerably firm, of about one to two inches, or two plugs of about one inch each, are used for the plugging of the flasks and test-tubes. An assertion such as that made by Dr. Williams at the British Association (Biological Section, September 1883), that cotton-wool plugs are not reliable, because they do not protect the fluids in the vessels plugged with them from accidental air-contamination, is to be accepted only as applying to very loose plugs and to cotton-wool not properly sterilised. To good firm plugs of sterile cotton-wool it evidently cannot apply, since all the results of all workers in this field (Pasteur, Sanderson, Cohn, Koch, Klebs, Buchner, and many others) are against it.

Instruments, such as the points of needles, and forceps, used in the processes of cultivation, lifting up cotton-wool plugs, making cotton-wool plugs, inoculations, &c., must be heated in the open flame of a Bunsen burner, if they are to be absolutely relied on for cleanliness. Scissors and knives used

for cutting tissues which are intended for inoculation, ought to be likewise scrupulously clean. One ought to keep a special set of instruments, the blades of which are capable of being heated in the open flame without being spoilt.

Syringes used for cutaneous, subcutaneous, or other inoculations, ought to be capable of being overheated. The ordinary Pravaz syringe of vulcanite not being capable of undergoing this process, Koch has devised a glass syringe similar to the Pravaz syringe. I do not use any syringe for inoculation, but prefer using each time a fresh *capillary glass pipette* made just before the inoculation. Into this pipette I draw the droplet to be used for inoculation, and having made a very small incision—about $\frac{1}{8}$ of an inch—through the skin, the pointed end of the pipette is pushed forward into the subcutaneous tissue for about half an inch or one inch and then the fluid is blown out into the tissue. In this way I am always absolutely safe from any contamination with a previously used virus, which might possibly adhere to one or other part of a syringe.

The fine point of capillary pipettes (Fig. 5), used for inoculation of animals, or for drawing out a drop of fluid of a cultivation in a flask or test-tube, or for inoculating material contained in a test-tube or flask, are thus made: while one hand holds the bulb of the pipette, the other holds one end, and putting at some distance from this end the tube into an ordinary flame and quickly drawing it out, a point of extreme fineness can be made. The same is done with the other end. Such a pipette can be considered as practically closed at both ends.

CHAPTER IV.

PREPARATION OF CULTURE-MEDIA FOR INOCULATION.

We have on a former page described the methods to obtain sterile stock of nourishing media suitable for artificial cultivations. The solids, as serum gelatine, serum, and hydrocele fluid, must, before solidification, be placed in test-tubes and small flasks, and then sterilised in the manner above described, to be made ready for establishing cultures, *i.e.* for inoculation. The Agar-Agar mixture however can, like broth, peptone mixture, beef extract solution, and gelatine mixtures, be kept as stock in large flasks. When thus sterile, these latter can be decanted into a number of test-tubes or small flasks, in which the cultivation is to be carried out. Gelatine mixtures (gelatine and broth, gelatine and peptone, gelatine and beef extract) and the Agar-Agar mixture, must of course be liquefied over a flame before being ready for decanting. The test-tubes most suitable are about six inches long, and should not be less than about one inch broad; the flasks are about of the capacity of one to two ounces, and ought to have a neck of comparatively good width. The test-tubes receive the fluids for about one and a half to two and a half inches in depth, the flasks for about one-fourth to one-third of their bulk. All these test-tubes and flasks with their cotton-wool plugs, before receiving the material, should be thoroughly sterilised by overheating. As I mentioned in the previous chapter, this ought to be well borne in mind, for starting with a sterile nourishing fluid—*i.e.* one that has been kept in the stock flask for several days to several weeks in the incubator at a temperature of from 32°—38° C. and that has remained perfectly clear and limpid—and working with thoroughly sterilised test-tubes and cotton-wool plugs—very

little care is required to obtain sterile material ready for inoculation. To start with a stock of nourishing material, however well sterilised, and to decant it into test-tubes with cotton-wool plugs not absolutely sterile must lead to failure. I have seen this happen over and over again, and all the material decanted became consequently contaminated and thereby useless for inoculations. The test-tubes and flasks must be well cleaned, then dried, placed in the air-chamber, and kept there exposed for several hours to a temperature of from 130°—150° C. on several successive days, or they may be thoroughly heated in all parts over the open flame of a gas-burner. The same applies to the cotton-wool, as mentioned in a former chapter. The test-tubes and flasks are plugged by means of clean forceps with the cotton-wool which is just brown, and then replaced in the air-chamber and again heated for several hours on two or three occasions up to a temperature of 130°--150° C., or they may be well heated over the open flame of the burner. To decant sterile stock fluid into these test-tubes and flasks, I proceed thus: A clean beaker with spout, covered with a clean glass plate, is placed on a wire net on a tripod over the flame of a Bunsen burner, and thoroughly heated for half an hour or so; then it is allowed to cool, and when cool, the plug of the stock flask is lifted with forceps, and some of the sterile fluid quickly poured from the flask into the beaker. The plug is replaced in the neck of the stock flask and the beaker covered with the glass plate. Of course the quantity poured into the beaker should be large enough to supply the required number of test-tubes or small flasks. The stock flask containing still some fluid, having been opened for however short a time, has of course been exposed to air-contamination, and therefore must be treated accordingly, if the fluid left in it is to serve as sterile nourishing material on a future occasion. Consequently it is subjected to boiling for from fifteen to thirty minutes, then placed in the incubator, boiled again the next day and put back in the incubator, where it is left at a temperature of from 32°—38° C. for several days. If after a week or so the fluid remains limpid, it is of course to be considered sterile.

Next, the fluid that has been poured into the beaker (covered with the glass plate) is poured as quickly as possible into the test-tubes, one after the other, by lifting with clean forceps the plug and pouring in the fluid to a depth of one and a half to two and a half inches, and the plug replaced.

During this procedure, contamination with air-organisms, if

there be any about, becomes inevitable. To lessen this chance as much as possible, it is necessary to lift the plug with clean forceps, to pour the fluid as rapidly as is practicable into the test-tube or flask, and to replace immediately the cotton-wool plug. Further it is necessary to bear in mind, that the atmosphere is not at all times and everywhere equally contaminated (see Prof. Tyndall's observations). I generally avoid undertaking this process on windy days, and when I do it, I generally close windows and doors and keep the air in the room as still as possible. I do not do it in a room in which

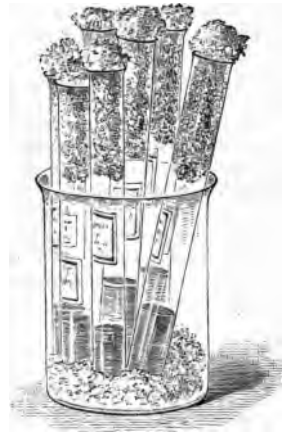


FIG. 6.—A BEAKER CONTAINING A NUMBER OF CULTURE-TUBES PLUGGED WITH COTTON-WOOL.

recently (say an hour or two previously) the floor, walls, or tables have been swept.

I have opened under these conditions the plugs of test-tubes containing sterile material, and kept them so for a time varying from one to ten seconds, and in some instances I have not seen more than from 10 to 20 per cent. contaminated.

Now, having filled the required number of test-tubes and flasks with the desired quantity of fluid, I subject these *seriatim* to boiling. By means of an ordinary test-tube

holder I hold them above a very small flame until the fluid boils, and keep it so boiling for from two to five minutes. During this process of boiling the cotton-wool is only slightly pulled up, and immediately before ceasing to boil the plug is again replaced, and pushed down with a clean glass rod. Then the test-tube is placed (of course upright) in a beaker at the bottom of which a layer of cotton-wool—a sort of cushion—has been placed. When finished, the test-tubes in the beaker are all transferred to the incubator and kept there for from twelve to twenty-four hours at a temperature of 32°—38° C. Then the boiling is repeated once more. After this they are kept in the incubator for several days to several weeks. I generally keep them there for two to three weeks, and all those in which the fluid has remained limpid and clear are considered sterile and ready for use. As a rule, starting with sterile stock fluid, and using thoroughly sterile test-tubes and cotton-wool plugs, after once or twice boiling after decanting, there ought to be no loss of tubes through accidental contamination with air-organisms (during decanting). Sometimes, however, I have had loss to the amount of 5 per cent. or more, but then there was always a hitch of some kind traceable. To decant under carbolic acid spray is not practicable and possesses many unpleasant drawbacks, besides, in some instances when I used it, there was really a greater percentage of contaminated tubes than without it. I therefore do not use the spray.

Test-tubes containing solid nourishing material are generally kept sufficiently inclined during solidification of the material (see a former chapter) to allow the material to spread into a layer of large area, although this is not essential.

CHAPTER V.

METHODS OF INOCULATION.

HAVING now in test-tubes and small flasks sterile material ready for inoculation, it is necessary to describe the mode of inoculating the same.

1. *Inoculations from Artificial Cultures.*—The first and simplest is the case where it is required to inoculate a new tube or flask with a definite organism that has been growing previously in a culture-tube; that is to say, where it is required to establish from an artificial cultivation a new and further artificial cultivation. Take a freshly drawn-out capillary pipette, with a fine point, as described in a former chapter; draw up with clean forceps slightly the top part of the cotton-wool plug of the old tube or flask, push carefully and gently one of the pointed ends of the capillary pipette—the other can be broken off blunt—through the remaining part of the cotton-wool plug, and push it downwards till it emerges into the culture-fluid, or, if this be solid material, till it reaches the spot or place where the organism is growing; allow a small droplet to ascend into the capillary pipette, which it readily does by capillarity; or if a larger quantity is required draw it up by gently sucking at the outer end of the capillary pipette. Then draw the capillary pipette altogether out of the tube and cotton-wool plug, and push this latter down with the forceps into its former position. Immediately after this proceed to inoculate the new culture-tube by doing exactly the same as before, viz., draw up slightly with the forceps the top part of its cotton-wool plug, push through the remainder of this plug the pointed end of the capillary pipette, *i.e.* the one containing the droplet of the material to be sown, and push it into the material at the bottom of the test-tube or flask. A trace of the sowing material flows out by itself, or, if a large

quantity is required, it is carefully blown from the pipette. If the sowing is to be carried out on the surface of the solid nourishing material, the inoculation is of course performed by depositing the seed on the surface; if in the depth, the end of the pipette is pushed down into the depth of the material and the seed there deposited. The pipette is then altogether withdrawn and the plug replaced as before. The new tube is then placed in a beaker on a cushion of cotton-wool, and exposed to the required temperature in the incubator.

If we have, however, a culture-fluid or any fluid that contains, as the microscopical examination proves, various species of organisms, which we wish to isolate, *i.e.* if we wish to introduce into a new culture-tube only one species, then the method of Klebs of "fractional cultivation," or the method of Lister and v. Nägeli of "dilution," is resorted to.

The "fractional cultivation" consists in the attempt to isolate by successive cultivations the different organisms that have been growing previously in the same culture. If we take up by means of a capillary pipette a trace of the culture-fluid, and inoculate with traces of it in the manner above described a series of new culture-tubes containing various nourishing materials, and expose these tubes in the incubator to a definite temperature, say 35° C., then the chances are that in the first twenty-four or thirty-six hours not all the different species of organisms sown out will have increased equally in numbers in all tubes; most probably only one species in each tube, *i.e* the one that grows best in this particular medium and at this particular temperature, will be found to have increased to an enormous extent, while the others have made little or no progress as yet. The nourishing fluid appears turbid, and filled chiefly with the one kind of organism. Now draw out with a fresh capillary pipette a minute droplet of this new culture and inoculate with a trace of it a new culture-tube. The chances are that you inoculate only one kind, that is, the one which is most abundant or perhaps is solely present. After twenty-four hours' incubation this new tube contains now probably only one kind of organism. To make it quite certain, inoculate from this a new culture-tube in the same manner, and now you probably have sown only a single species. In this manner by continued transference it is possible to obtain cultures with only one species of organisms. Many conditions, such as naked-eye appearances of a particular kind, coloration of the culture-medium, formation of a pellicle, the quantity of growth in a given time, soon indicate whether we have the desired single

species; in some instances it is, however, extremely difficult to isolate after this method.

The method of "dilution" means diluting the culture-fluid containing the various species to a very large extent with some sterile indifferent fluid, such as well-boiled saline solution of 0·6 per cent., and then inoculating new tubes with a droplet of this greatly diluted material. For this purpose draw into a rather large pipette a tiny droplet of the old culture-fluid, then pass the pointed end of this pipette into a test-tube or flask (plugged) containing well-boiled saline solution, and draw up a quantity of this solution so as to greatly dilute (1000-fold or more) the droplet of culture-fluid, and with this inoculate then a series of new culture-tubes containing different nourishing material, using always only a trace for inoculation. In this way it is probable that, owing to the great dilution, the trace of a droplet of this mixture used for the new inoculation contains only one species. Using a series of new culture-tubes and inoculating them thus, after twenty-four hours of incubation it will be found that some tubes have not received any seed, others only one species. If it be required to dilute the original fluid greatly, say if it teems with different organisms, then a droplet of this is placed into a large flask containing the well-boiled saline solution, so that a dilution of 1 in 1,000,000 or more can be effected.

The two methods *i.e.* that of fractional culture and of dilution, may be successfully combined in this way: from the first or second new culture, established after the method of fractional cultivation, in which after twenty-four or thirty-six hours one species greatly predominates, draw out with a large capillary pipette a droplet, and dilute this to a great extent with the saline solution, as described above, and now inoculate with a trace of this mixture a new culture-tube. Or, if after twenty-four hours' incubation the microscope reveals in this further culture more than one species, continue the process of dilution and inoculation for a further generation. Thus it is possible to obtain cultures of only one species, although the original fluid contained several species of organisms.

2. *Inoculations with Blood, Juices, and Tissues.*—To establish a cultivation from blood of a dead animal, cut open the thorax by removing the sternum with clean scissors, cut open the pericardial sac, pierce with the pointed end of a fresh capillary pipette the wall of the right ventricle or right auricle, and allow a drop or two of blood to ascend into the pipette, or if a larger quantity is required suck it up. Withdraw the pipette and inoculate new culture-tubes as above. Or, if blood of a

large vein is required, separate the vessel with clean instruments, and make a small incision with clean scissors and push the pointed end of the capillary pipette well forward. If juice of a lymphatic gland, or spleen, or other parenchymatous organ be required, pierce the organ after having washed its surface with solution of perchloride of mercury (Koch), with the pointed end of a capillary pipette, then push it into the part required for a little distance, and squeezing the organ press a drop or two of the juice into it. The same procedure is adopted when the pus of an abscess is required, the wall of which can be pierced with the pointed end of the capillary pipette. If not, a slight incision is made and the pipette introduced through this into the abscess. If blood of a living animal is required, expose a vessel with clean instruments, make a small incision with clean scissors, push through this incision the pointed end of the capillary pipette well forward, and allow the blood to rise into the capillary tube. If blood of a living human being is required, clean well with soap and water and then with strong carbolic acid or perchloride of mercury solution the tip of a finger, make a venous congestion in the last phalanx by compressing it with a corner of a hankerchief, prick the volar skin of the phalanx with a clean (overheated and cooled) needle, and plunging the pointed end of the pipette into the drop of blood allow a droplet to ascend into the capillary tube of the pipette.

If solid tissues or parts of tissues are required, *e.g.* the base of an ulcer, a tubercle of the liver, spleen, or lung, it is possible to squeeze into the capillary tube of a pipette, after pushing its pointed end into the part, a small droplet of juice of the part required; but if this be not practicable, *i.e.* if a solid particle be required, then follow Koch's method. This is as follows: cut with clean scissors or scalpel into the part, dig out rapidly with the point of a needle or platinum wire previously overheated in the flame of a burner a small particle, and quickly introduce this into the culture-tube to the place required, *e.g.* surface or depth of a solid or fluid nourishing material. Of course in this case the cotton-wool must be altogether lifted, and therefore contamination with organisms is possible. But inoculating several tubes at once and performing the operation quickly, one always succeeds in getting some of the tubes without any air-contamination. I have made numerous inoculations with solid particles (tubercles) in this manner, and like Koch have seen only a small percentage of tubes going bad through contamination with air-organisms.

The same plan, *i.e.* of using the clean point of a needle or

platinum wire for taking up the material to be used for inoculation, is resorted to if one has to deal with the culture of solid nourishing material, on or in which the organisms are growing that we want to transplant either for inoculation of a new tube or of an animal. A useful method, which does not require the lifting out of the plug at all, and which can easily be employed in the last case, is this: deposit from the pointed end of a capillary pipette a droplet of some sterile fluid (broth or thoroughly boiled saline solution) on the spot of the solid medium on which the organisms are growing, then scratch this spot with the end of the capillary pipette in order to get the organisms off from the solid basis and mixed with the drop of fluid deposited there, then let this drop again ascend into the end of the capillary pipette, and withdraw this altogether. All this can be done without lifting out the cotton-wool plug of the test-tube or flask in which the growth is proceeding.

If one has to use a particle of tissue the surrounding portions of which are probably contaminated by putrefactive organisms, *e.g.* a tubercle in the lung or a tubercle in the spleen, it is well to follow Koch, and to disinfect the surrounding parts by just washing them with a dilute solution of corrosive sublimate, and then to remove these parts with clean scissors so as to obtain the central particle which one wishes to use for inoculation: of course one must not steep the whole organ in sublimate solution, since this would naturally destroy all organisms.

All these methods can be easily modified according to the requirements of the special cases, and it is not necessary here to give more than what has already been described in the preceding.[1]

In order to observe in a microscopic specimen the gradual changes in the growth of a micro-organism, there are several methods employed. In all of them it is of course necessary to keep the specimen heated up to the desired temperature.

The simplest method consists in sowing the organisms on a suitable nourishing material in a small glass cell, fit to be placed on the stage of a microscope and to be there observed even with high powers, similar to those cells which Koch has used in his studies on bacillus anthracis. Such a glass cell consists of a glass slide, in its centre a concave pit, not too large, and capable of being quite closed up by an ordinary cover-glass, the edges of which fasten by means of clean paraffin or olive oil. Place with a clean needle a speck of spleen pulp

[1] Compare also Koch, *Untersuchungen über pathogene Bacterien*, in *Berichte aus dem k. Gesundheitsamte*, Berlin, 1881; and *Die Aetiologie d. Tuberculose*, *Berlin. klin. Wochenschrift*. No. 15, 1882.

and the peritoneal exudation, contain the same bacilli as the subcutaneous exudation. Many of them include spores. By injecting the bacillus into the peritoneal cavity of guinea-pigs death is produced rapidly, especially after passing it through two generations, so rapidly indeed that the animals often die within sixteen hours (Burdon Sanderson and Klein). In all these instances a viscid transparent slightly but spontaneously coagulable exudation, poor in white and red corpuscles, is found in the peritoneal cavity, and the peritoneum of all parts is highly inflamed. Bacilli are present in it in enormous numbers, many of them containing spores. The blood of the heart does not contain bacilli immediately after death, but has them some hours after.

The bacilli in question are about 0·003 to 0·005 mm. long and a little over 0·001 mm. thick; they are rounded at their ends; they form chains of two and more, and these chains are straight or broken. They also form leptothrix, straight, or more commonly curved. The bacilli have been artificially cultivated by Pasteur[1] in blood-serum and in neutral solution of Liebig's meat extract. Gaffky[2] grew them on potatoes at 38° C. The artificial culture is capable of producing the malignant œdema, but it is always necessary to inject more than minimal quantities. The bacilli grown in fluids outside and inside the body form spores without free supply of air, and are therefore anaërobic (Pasteur).

In human fæcal matter there are always present innumerable masses of bacteria—micrococci, single and in dumb-bells, and in clumps of zooglœa, bacterium termo, and various species of bacilli, varying in thickness, length, and in motility, some being motile, others not. It has been shown by Bienstock (*Centralbl. f. med. Wiss.* 1883, p. 949) that a bacillus can be cultivated from normal human fæces which in many respects resembles the bacillus of malignant œdema; it produces death in mice, but without the symptoms of malignant œdema.

Professor Rossbach has maintained (*Centralblatt f. d. med. Wiss.* 5, 1882) that when a solution of papayotin (the juice of *Carica papaya*) is injected into the veins of a rabbit, the animal dies, and shortly after death—even so short a time as fifty minutes after the injection—there are found in the blood large numbers of bacteria. Dowdeswell, however, states (*Practitioner*, May, 1883) that solutions of papayotin contain as a rule the spores of a motile bacillus which in all respects resembles bacillus subtilis; in artificial cultures in 10 per cent solutions of papayotin, in blood-serum, and in broth, these spores develop into bacilli which form leptothrix filaments, and in them spores soon make their appearance. Filtered papayotin solutions, when injected into the blood of rabbits, kill like unfiltered ones, but neither during life nor after the death of the animals could any organisms be detected in the blood. It appears to follow from these experiments, that papayotin solutions contain spores, and that these spores are those of a bacillus subtilis which does not possess any specific pathogenic properties.

(*k*) *Bacillus of symptomatic anthrax* (Ger. *Rauschbrand;* Fr *charbon symptomatique*, Arloing, Cornevin, and Thomas,

[1] *Bull. de l'Acad.* 1877. [2] *Mittheil. a. d. k. Gesundh.* 1880.

Bull. de l'Acad., 1881 ; Eng. *black leg, quarter-evil*). This disease, which is not uncommon in cattle, generally ends fatally and is very infectious. It is characterised by hæmorrhagic effusion (or "tumour") in the subcutaneous and intermuscular tissues of one or other, or both, anterior or posterior extremities, in consequence of which the movements of the animal so affected become greatly impeded. The animals generally die in the course of the second or third day after infection. The subcutaneous tumour contains numerous bacilli, as do the abdominal and thoracic viscera.

The bacilli are about the size of those of malignant anthrax or a little thicker ; they are rounded at their ends and often include at one end a bright oval spore ; this is also present in the bacilli of the parenchymatous organs (as will be shown below, this never occurs in the bacillus of malignant anthrax). The bacilli are either single or form short chains. Some of the bacilli are motile.

Inoculations with them into the subcutaneous tissue of guinea-pigs, rabbits, sheep, and calves always prove fatal, the same subcutaneous hæmorrhagic effusions being produced.

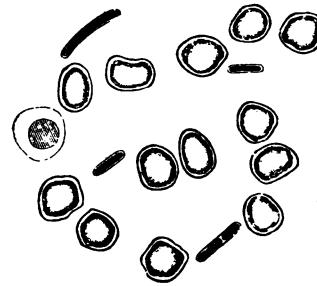


FIG. 72.—BLOOD OF A GUINEA-PIG DEAD OF SYMPTOMATIC ANTHRAX.
Blood-corpuscles and between them several bacilli.
Magnifying power 700. (Stained with Spiller's purple.)

Injections of small quantities of bacillus-containing material into the veins produces only a slight febrile disorder ; large doses produce death. Animals in which by intravenous injection of small doses slight illness has been produced are afterwards protected against the fatal dose. But minimal doses injected subcutaneously also produce only a slight

transitory swelling, and the animal so treated is afterwards protected against the fatal dose (Arloing, Cornevin, and Thomas). The spores of the bacilli when heated up to 85° C. for six hours lose their virulence (Arloing, Cornevin, and Thomas).

(*l*) *Bacillus anthracis.*—Pollender,[1] Brauell,[2] Davaine,[3] and then Bollinger[4] recognised in the blood of animals dead of malignant anthrax the presence of stiff short and long rods, which Davaine called *bactéridie du charbon.* They were identified by Cohn[5] as bacilli in morphological respects similar to bacillus subtilis, except that the bacilli anthracis are non-motile.

Koch[6] showed the ubiquitous distribution of these bacilli in the blood of the organs, and especially of the spleen. He

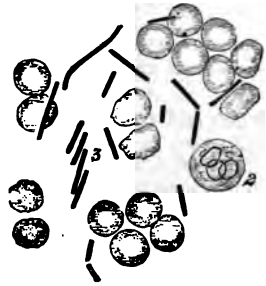


FIG. 73.—HEART'S BLOOD OF A MOUSE DEAD OF ANTHRAX.

1. Blood-discs.
2. White blood-corpuscle.
3. Bacilli anthracis.

Magnifying power 700. (Fresh specimen.)

succeeded in cultivating these bacilli artificially, taking a bit of spleen of a mouse (which animal is very susceptible to fatal anthrax), and watching the growth of the bacilli in a microscopic specimen. He saw that the rods multiply by division, and that they grow into long, homogeneous-looking, straight or twisted filaments in which after some time, and with free access of air, bright oval spores make their appearance, while the filaments become homogeneous and swollen. These spores become free, and when artificially cultivated or injected into a rodent animal, germinate into the characteristic bacilli;

[1] *Vierteljj. f. gericht. Med.* 1855.
[2] *Virchow's Archiv*, vol. 14, 1858.
[3] *Comptes Rendus*, lvii. 1863.
[4] *Med. Centralblatt*, June, 1872.
Beitr. z. Biol. d. Pflanzen, vol. ii.
[6] *Ibid.* vol. iii.

but not by feeding. He also found[1] in twenty out of twenty-one healthy pigs examined, the actinomyces present in the crypts of the tonsils.

Israel[2] succeeded in transmitting the disease to a rabbit by inserting into the peritoneal cavity a piece of a human antinomyces-tumour.

R. Virchow quite recently,[3] in conjunction with O. Israel and Duncker, ascertained that pork occasionally contained whitish chalky nodules, larger than those due to trichinæ, and containing in their interior the actinomyces.

O. Israel[4] claims to have succeeded in artificially cultivating the actinomyces on solid ox-serum ; in fluid media the growth does not succeed, owing to the swelling up and death of the actinomyces-corpuscles.

[1] *Centralbl. f. med. Wiss.* 15, 1881.
[2] *Ibid.* xxvii. 1883.
[3] *Virchow's Archiv*, vol. xcv. p. 544
[4] *Ibid.* vol. xcv. p. 142.

CHAPTER XVII.

ON RELATIONS OF SEPTIC TO PATHOGENIC ORGANISMS.[1]

THERE is hardly any question which to the pathologist and sanitary officer can be of greater importance than the relation of septic to pathogenic organisms. To the pathologist the life history of a micro-organism, outside and within the animal body, must ever remain an important field of inquiry; to the sanitary officer all conditions affecting the life and death of those organisms which produce, or at least are intimately bound up with, infectious diseases, such as the distribution and growth of these micro-organisms outside the animal body, the agencies which affect it in a favourable and unfavourable sense, are the points which he has particularly to consider in dealing with the spread and prevention of infectious maladies. Now, it is known of many micro-organisms, both those that are associated with putrefactive processes as well as those that are bound up with infectious disease, that temperature, the medium in which they grow, presence and absence of certain chemical compounds are capable of materially affecting them. I need not for this purpose enumerate all that is known already by direct experiment, but will only limit myself to reference to the researches of Schröter, Cohn, and Wernich on that group of micro-organisms known as pigment bacteria, *i.e.* bacteria which only under certain conditions, notably temperature and soil, produce definite pigments (Cohn's *Beiträge zur Biologie d. Pflanzen*); to those of Hansen (Carlsberg Laboratory) on yeast; to those of Neelsen on the bacilli producing the blue colour of milk, the bacillus syncyanus (*Beitr. zur Biol. d. Pflanzen*, iii. 2, p. 187); to the works of Toussaint, Pasteur, Chauveau, Koch,

[1] The greater part of this chapter is copied from an interim report by myself to the Medical Officer of the Local Government Board, 1884.

and others on the bacillus anthracis; Arloing, Thomas, and Cornevin on the bacillus of symptomatic charbon; of Koch on the bacillus of tuberculosis; of Israel on actinomyces, and many others; and particularly would I refer to the many valuable suggestions and considerations expressed by v. Nägeli in these respects in his book, *Die niederen Pilze*, München, 1877 and 1882.

While from these observations it would appear that both septic and pathogenic micro-organisms are capable of suffering some modifications in their morphological and physiological behaviour, it is nevertheless still an open question whether an organism which under ordinary conditions is only associated with putrefactive changes in dead organic material, and which cannot under these ordinary conditions grow and multiply within the living body, can, under certain extraordinary circumstances, become endowed with the power of growing and multiplying within the body of a living animal, creating there a pathological condition, inducing there an infectious disease.

Three distinct septic micro-organisms have, after numerous experiments and careful observations, been mentioned, as being capable when growing under certain extraordinary conditions of assuming pathogenic properties. These three organisms are: (*A*) the common bacillus of hay infusion is said by Buchner to be capable of transformation into bacillus anthracis; (*B*) a bacillus subtilis, present in the air, which, although quite harmless in itself, assumes distinct pathogenic properties when growing in an infusion of the seeds of *Abrus precatorius*, becoming hereby endowed with the power of causing severe ophthalmia (Sattler); (*C*) a common mould, aspergillus, which harmless in itself, when grown on neutral and alkaline material at about body-temperature (38° C.) assumes, according to Grawitz, very poisonous properties, producing in rabbits inoculated with it death, with metastatis of aspergillus and its spores in the various internal organs.

There are in the literature of micro-organisms other cases mentioned, in which such a transformation has been *supposed*, but without any experimental proof, and we need not therefore trouble ourselves more about them.

Let us now review *seriatim* the above three cases:

(*A*) Dr. Hans Buchner in a paper, which for many reasons may be considered an important one, "Ueber d. experim. Erzeugung des Milzbrandcontagiums, &c.," published in the *Sitzungsberichte d. math. physik. classe d. k. Bair. Akademie d. Wiss.* 1880, heft iii. p. 369, states that he succeeded in

transforming the common bacillus of hay infusion, the hay bacillus, into the bacillus anthracis.

The hay bacillus and the bacillus anthracis rank together morphologically under that form which Cohn has named bacillus subtilis.

The two are, however, not quite identical in morphological respects. The hay bacillus is a minute rod or cylindrical-shaped bacillus, which by elongation and division produces chains and further threads just like the bacillus anthracis, but in the latter (*i.e.* bacillus anthracis) the bacilli and their threads are composed of cubical elements, as is shown in stained specimens, and as has been mentioned in a former chapter, whereas in those of the hay bacillus the elements are rods or cylinders. I have seen, however, many of the short hay bacilli which being constricted, *i.e.* in the act of division, appear as two short more or less cubical elements placed end to end. It is generally assumed that in hay bacillus the bacilli are always rounded at their ends, whereas the bacilli anthracis are as if straight cut at their ends; but this is not universally the case, since I have seen in cultures the bacilli anthracis with distinctly rounded ends. But, speaking generally, the hay bacillus is a rod more distinctly rounded at its ends, the bacillus anthracis of the blood is not so.

The bacillus anthracis is slightly thicker than the hay bacillus. In artificial cultivations carried on in neutral broth the bacillus anthracis is about twice as thick as the hay bacillus growing in the same fluid, and when both are growing in neutralised hay infusion the two are very conspicuously different from one another, and can at a glance be distinguished from one another; the hay bacillus being about half the thickness of the bacillus anthracis. In stained specimens, too, the latter is beautifully made up of a row of cubical cells, whereas the former consists of cylinders only.

True, the bacillus anthracis is not always of the same thickness, for, as I have shown, when growing in neutral pork broth it is decidedly thicker than in the blood of an animal dead of anthrax. And also in the blood of different animals the bacillus anthracis slightly varies in thickness, for in the guinea-pig's blood it is slightly thicker than in that of the rabbit or sheep.

The hay bacillus is motile, possessed of a flagellum, and therefore capable of locomotion; the bacillus anthracis is not motile. I am quite aware that Cossar Ewart (*Quarterly Journal of Microscopical Science*, April 1878) states that he has seen in a specimen kept artificially heated under micro-

scopic observation that the bacillus anthracis, at first non-motile, is capable of becoming motile. At one or both ends a flagellum grows out from its body. But this observation is unreliable, since Ewart did not guard himself in any way from the accidental introduction of septic bacilli, many of which are motile. Besides, he says of the bacilli, which he figures as anthrax bacilli, that they are connected with one another by two fine threads, and that they probably separate from one another and each retains one filament, which is its flagellum. But his observations, so far as they have application to anthrax bacilli, are capable of quite a different interpretation. In every specimen of blood and in every artificial culture bacilli can be seen, in which at one place or more the protoplasm is wanting, owing as I have shown, to degeneration; in such places only the empty sheath is present, and of course in fresh specimens this gives the appearance as if the two protoplasmic portions of the bacillus were connected with one another by two fine threads, *i.e.* the sheath being transparent is seen here edgeways.

In no instance has the bacillus anthracis been observed to be motile. I have examined thousands of specimens of fresh bacillus anthracis in the blood and in artificial cultures, and I have never seen anything that in the least would lead me to differ from this proposition.

As regards the spores they are of the same aspect and size in both the hay bacillus and bacillus anthracis. The threads in good cultures form in both cases the same bundles more or less twisted and forming convolutions, but in certain cultures of the bacillus anthracis, *e.g.* in broth, in which the growth is limited to the bottom of the fluid, the convolutions and the twisted condition of the threads are more pronounced, more cable-like.

Hay bacillus being motile, every culture of it is uniformly turbid, the bacilli being capable of moving about, and after a day or two of incubation at 35° C. they form a distinct pellicle on the surface of the fluid, which in further stages becomes complete and thick. These pellicles are composed of a dense feltwork of threads of the bacilli, and in them spore-formation is going on.

By shaking the fluid the pellicle sinks to the bottom, and if the fluid is not exhausted yet, a new pellicle is formed of the same nature.

In no culture of hay bacillus are there ever observed those cloudy, fluffy, whitish and transparent convolutions that are seen in cultivations of bacillus anthracis carried on at the

bottom of fluid broth, and which have been so accurately described by Pasteur.

Both the hay bacillus and the anthrax bacillus when growing on gelatine mixtures liquefy the gelatine; both when growing in meat broth turn the at first colourless fluid in the course of incubation to an amber, and further to a brown, tint.

The hay bacillus is capable of thriving well in acid solutions, it grows copiously in hay infusion, which is of a distinct acid reaction; the bacillus anthracis, although capable of making a slight progress in acid hay infusion, does not get far, for degeneration soon sets in; it thrives best in neutral solutions. Hay bacillus thrives also very well in neutral solutions.

Buchner states, that by successive cultivation of bacillus anthracis under *constant variation of the nutritive material*, he saw it assume gradually the properties of hay bacillus. Thus he saw that its mode of growth gradually changed, inasmuch as instead of forming, as the typical bacillus anthracis does, fluffy convolutions at the bottom of the fluid-nourishing medium, it gradually showed a tendency to stick to the glass and to the surface of the fluid, and to form a sort of pellicle just like the hay bacillus does. This I consider to be an erroneous interpretation of an easily explained and simple fact. It does not want any of the many successive generations of bacillus anthracis, in which Buchner says he has achieved this transformation, it simply requires two nourishing fluids, in both of which the bacillus anthracis will thrive well, but which fluids differ in specific gravity. Let Buchner do as I have done, let him take two test-tubes, both containing sterile broth, but in one the broth concentrated, in the other dilute. Let him inoculate the two test-tubes with bacillus taken from the same blood, say of a guinea-pig dead of anthrax, let him place them in the incubator at a temperature of 35°--42° C. After two or three days, and more decidedly later, he will notice this very difference in the aspect of the cultures that he lays so much stress on as indicating a change in the physiological character of the bacillus. One test-tube, containing the dilute broth, shows the typical fluffy convolutions at the bottom of the fluid; while the other, containing concentrated broth, shows a distinct attempt at the formation of a pellicle. Let him now take out a droplet from this second test-tube and inoculate with it two test-tubes of the same nature as above, *i.e.* one containing concentrated broth, the other dilute broth. After two or three or more days of incubation he will find exactly the same differences as above.

Buchner states that the bacillus anthracis when carried

On careful analysis, it will be found that it is not capable of explaining all the facts of the case. As we mentioned in a former chapter, cattle inoculated with blood of a guinea-pig dead of anthrax become affected with anthrax, which, although not fatal, is nevertheless sometimes very severe. The animal recovers, and is now, for a time at least, protected against a second attack. But there is absolutely no ground for the assumption that if an infusion of the tissues of this animal were made, the bacillus anthracis sown in it would not thrive luxuriantly, seeing that bacillus anthracis grows on almost anything that contains a trace of proteids. Similarly when of the tissues of a guinea-pig, or mouse or rabbit, dead of anthrax, an infusion is made, and this is used as nourishing material for bacillus anthracis in artificial cultures, it is found that these latter thrive splendidly. The same fact I have observed in the case of swine-plague. There is then no reason whatever for assuming that, after one attack of illness, the blood and tissues become an unfavourable soil for a second invasion of the same organism, and that this should be due to the exhaustion of some necessary chemical compound.

There is another theory, commonly spoken of as the *Antidote Theory* (Klebs). According to this, the organisms growing and multiplying in the body during the first attack produce, directly or indirectly, some substance which acts as a sort of poison against a second immigration of the same organism. I am inclined to think that this theory is in harmony with the facts. There is nothing known, from the observations before us, which would negative the possibility of the correctness of this theory; nay, I would almost say all our knowledge of the life of the micro-organisms points to the conclusion that the different species are associated with different kinds of chemical processes, and that as a result of the activity we find different chemical substances produced.

The different fermentations connected with the different species of fungi afford striking illustrations of this view. According to this theory, we can well understand that—just as in the case of an animal, say a pig, unsusceptible to anthrax—the unsusceptibility being due to the presence in the blood and tissues of a particular chemical substance inimical to the growth of the bacillus anthracis—so also in the case of a sheep or ox that has once passed through anthrax—there is now present in the blood and tissues a chemical substance inimical to the growth and multiplication of the bacillus anthracis whereby these animals become possessed of immunity against a second attack of anthrax.

Whether this chemical substance has been elaborated directly by the bacilli, or whether it is a result of the chemical processes induced in the body by the bacilli during the first illness, matters not at all; it is only necessary to assume that the blood and tissues of the living animal contain this chemical substance.

Some observers (Grawitz, &c.) are not satisfied with this theory, but assume that owing to the first attack the cells of the tissues so change their nature that they become capable of resisting the immigration of a new generation of the same organism. There is absolutely nothing that I know of in favour of such a theory; it is impossible to imagine that the cells of the connective tissues, of the blood and of other organs, owing to a past attack of scarlatina, become possessed of new functions or of some new power, as, for instance, a greater power of oxidising or the like. Connective tissue-cells, blood-corpuscles, liver-cells, and other tissues are, so far as we know, possessed of precisely the same characters and functions after an attack of scarlatina as before.

On the whole then, we may, it seems, take it as probable, that owing to the presence in the normal blood and tissues in a living animal of a chemical substance inimical to the growth of a particular micro-organism, this animal is unsusceptible to the disease dependent on the growth and multiplication of this micro-organism; and further, that in those infectious maladies in which one attack gives immunity against a second attack of the same kind, one attack produces a chemical substance in the blood and tissues which acts inimically to a new immigration of the same organism; hence the animal becomes unsusceptible to a new attack, or is "protected." This is not the case with all infectious maladies, for, as is well known, in a good many instances a single attack does not protect against a second; and, as is also well known, a first attack may protect but only for a limited period, or for a period greatly differing in different individuals. All this would be explained by our theory in the same way as it is explained by the other theories; viz., when one attack does not protect, no inhibitory chemical substance has been produced; while in those diseases in which one attack does protect only for a limited period, the necessary inhibitory substance has only lasted for a limited period, and so on.

CHAPTER XXI.

ANTISEPTICS.

In former chapters we have on several occasions mentioned that a variety of substances and conditions are capable of exerting a detrimental influence on the life and growth of micro-organisms. Amongst these are—The presence of certain substances in the nutrient soil, the temperature, and some chemical products, such as those belonging to the aromatic series, phenol, indol, skatol, &c. The presence of certain substances in the nourishing material is, as we have seen, an essential condition, *cæteris paribus*, for the growth and multiplication of micro-organisms. Thus pathogenic organisms cannot thrive in an acid medium, they cannot thrive if proteids or allied compounds and certain inorganic salts are absent; putrefactive and zymogenic organisms, on the other hand, or, at any rate, some of them, are capable of thriving well in acid media (*e.g.* the bacillus subtilis in acid hay-infusion, the micrococcus ureæ in acid urine). Further, many (not all) pathogenic organisms cannot thrive unless they are exposed to a certain degree of warmth; they thrive best at blood-heat, while putrefactive and many zymogenic organisms thrive well at ordinary temperatures, though of course their growth is more rapid at higher temperatures, such as 30°—38° C. Heat above 50° or 60° C. arrests the growth of and even kills many organisms, except the spores of bacilli, which, as we find on a former page, survive even when exposed to the temperature of boiling water for several minutes. The presence of carbolic acid, phenol, thymol, salicylic acid, perchloride of mercury,

&c., prevent even when in great dilution the growth of micro-organisms.

In any inquiry into the influence of one substance or another on micro-organisms it is necessary to bear in mind that the influence of certain conditions on the micro-organisms may be a twofold one: (1) the condition may be unfavourable to the growth of the organism in question, and (2) the condition may be fatal to the life and existence of it. The second condition involves, *a fortiori*, the first; but the reverse is not the case. Owing to the failure to distinguish between these two propositions a great deal of confusion has arisen on the subject. One hears constantly this or that substance is an "antiseptic," meaning by this a substance inimical to the growth of micro-organisms, or a substance is a "germicide," implying by this that this substance kills the organisms; but when one comes to analyse the observations that are said to establish this reputation for a particular substance, one finds that the substances in question are really only detrimental to the growth of the organisms.

By sowing any micro-organism into a nourishing medium, to which has been added a certain substance (*e.g.* carbolic acid to the amount of 1 per cent.), and exposing this medium to the conditions of temperature, moisture, &c., otherwise favourable to the growth of the organism, if we find that after the lapse of a due period the growth is retarded or altogether inhibited, the conclusion is drawn that this substance (viz., the carbolic acid of 1 per cent.) is an antiseptic. There is nothing more fallacious than this method of reasoning; a great many micro-organisms can be exposed to a 1 per cent. solution of carbolic acid for hours without in the least being affected, for on being then transferred to a suitable nourishing medium they grow and thrive well. Similarly by placing the spores of bacillus anthracis in a proteid medium containing perchloride of mercury of the strength of 1 in 300,000, it is found (as Koch has shown) that the spores are absolutely incapable of germinating. But if from this the conclusion is drawn, that perchloride of mercury of the strength of 1 in 300,000 is a germicide, I should most strongly dissent, for perchloride of mercury even of the strength of 1 per cent. is not a germicide any more than vinegar; for on placing the spores of bacillus anthracis in a proteid medium, to which so much vinegar or any other acid has been added as makes it decidedly acid, it will be found that the spores do not germinate.

In order to pronounce a certain substance an antiseptic in the strict sense of the word, it is necessary to place the organisms in this substance for a definite time, then to remove them thence, and to place them in a suitable nourishing medium; if they then refuse to grow the conclusion is justified that the exposure has injured or destroyed the life of the organisms. In the case of pathogenic organisms a substance to be pronounced a germicide must be shown to have this power, that when the organism is exposed to the substance and then introduced into a suitable artificial medium it refuses to grow; and it must also be shown that when introduced into a suitable animal it is incapable of producing the disease which the same organism, unexposed to the substance in question, does produce.

I have made a good many observations on the influence of antiseptics on micro-organisms, both putrefactive and pathogenic, and I have found that many assertions hitherto made on this subject, treated in the above light, are absolutely untrustworthy and erroneous.

Various species of putrefactive micrococci, bacterium termo, bacillus subtilis, various pathogenic micro-organisms, as bacillus anthracis, bacillus of swine fever, absolutely refuse to grow in media to which is added phenyl-propionic acid, or phenyl-acetic acid, to an amount so small as 1 in 1,600; but if the same organisms are exposed to these substances in much stronger solutions, 1 in 800, 1 in 400, or even 1 in 200, and then transferred to a suitable nourishing material, it is found that they have completely retained their vitality, they multiply as if nothing had been done to them. I have exposed the spores of bacillus anthracis to the above acids of the strength of 1 in 200 for forty-eight hours and longer, and then inoculated guinea-pigs with them, and I found that the animals died of typical anthrax in exactly the same way as if they had been inoculated with pure spores of the bacillus anthracis.

Koch has published a large series of systematic and most valuable observations[1] made in testing the influence on spores of bacillus anthracis of a large number of antiseptics (thymol, arsenate of potassium, turpentine, clove-oil, iodine, hydrochloric acid, permanganate of potassium, eucalyptol, camphor, quinine, salicylic acid, benzoic acid, and many others), and amongst them he found perchloride of mercury to be the most powerful, since even a solution of 1 in 600,000 is capable of impeding, one of 1 in 300,000 of completely checking, the germinating

[1] *Mittheil. aus d. k. Gesundheitsamte*, Berlin, 1881.

power of the spores. To regard these substances, from these observations, in any way as antiseptics for the spores of bacillus anthracis would be no more justifiable than to consider weak vinegar as such. Perchloride of mercury in a solution of 1 in 300,000 is no more capable of interfering with the life and functions of the spores of bacillus anthracis than water or salt solution, for the spores may be steeped in this solution for any length of time, and yet on being transferred to a suitable medium they grow and multiply splendidly, and when inoculated into rodents they produce fatal anthrax with absolute certainty. With my friend Dr. Blyth, Medical Officer of Health for the Marylebone district in London, I have tried the action of a number of substances in common use as antiseptics (*e.g.* Calvert's fluid, pure terebene, phenol 10 per cent., perchloride of mercury 1 per cent.) on the spores of bacillus anthracis, exposing these in comparatively large quantities of the above fluids (the two being well mixed) for twenty-four hours, and then inoculating guinea-pigs with them (spores and antiseptic). The animals died with symptoms of typical anthrax, the blood teeming with the bacillus anthracis. These substances then are no more antiseptics, and still less germicides for the spores of bacillus anthracis, than water is.

In all these inquiries, particularly in those upon pathogenic organisms capable of forming spores, the influence of the substances must be judged not merely by their action on the organisms, but also on the spores; for, in this very case of the bacillus anthracis, the bacilli taken from the blood of an animal dead of anthrax are killed after an exposure of say ten minutes to a solution of phenyl-propionic acid of the strength of 1 in 400, or even 1 in 800, whereas the spores of the bacilli (produced in artificial cultures) withstand completely exposure to this acid of any strength and for any length of time.

It is not my object to pass here in review all that has been done in this interesting field of research, important because of its obviously great practical importance. The work hitherto done has been enormous, but, I fear, of less utility than at first sight appears, for in most of it the point most prominent in the mind of the worker was to ascertain whether the particular antiseptic, mixed with the nourishing medium in a solution of definite strength, has or has not the power of inhibiting the growth of the micro-organisms. This point no doubt is of some interest, and perhaps of great interest, but whether a particular substance is an antiseptic in the proper sense of the word, *i.e.* whether on exposing the organisms to

this substance in a solution of definite strength and for a definite peroid, the organisms become afterwards incapacitated from growing or multiplying; or still more, whether or not the substance is a germicide, *i.e.* is capable of altogether annihilating the life of the organisms; these are questions which require special attention, and represent a wide and rich field of inquiry; but, as far as I can see, it has received only in very few instances due attention.

APPENDIX TO CHAPTER XI.

A VERY useful method for the isolation of a mixture of bacteria is the one now extensively used by Koch and his school, it is this: liquefy over gentle heat sterile gelatine mixture (gelatine and peptone, or gelatine, peptone, and broth, as mentioned on p. 15) contained in a sterile plugged test-tube, then inoculate this with the smallest trace of the bacterial mixture, either by capillary pipette or by a platinum wire, shake it so as to distribute the introduced bacteria; then pour this liquid gelatine mixture in a thin layer on sterile glass plates, or flat watch-glasses or any glass vessel, and keep this in a closed chamber (a bell-glass or any other vessel fixed by olive oil or lard to a glass plate) kept moist by wet filter-paper. Keep this in the temperature of the room, or in the incubator, up to a temperature of 20° C., so that the gelatine layer remains solid. After several days a number of isolated spots will be noticed, each of these spots owing their origin to the multiplication of a bacterium. Some of these spots contain only one species, and from these pure cultivations in test-tubes can then easily be established. In this way Koch easily succeeded to isolate the comma-bacilli present in the mucus-flakes of the choleraic intestine, a small particle of such a flake being distributed in the liquefied gelatine mixture contained in a test-tube.

Another method of isolation is one similar to that described on p. 31: Liquefied sterile gelatine mixture, or liquefied sterile Agar-Agar mixture, is poured in a thin layer on the glass plates, watch-glasses, or glass-cells previously sterilised, and then kept in a moist chamber as above. When the mixture is set, draw on its surface lines with a capillary tube or platinum wire dipped in the bacterial fluid.

The comma-bacillus of Koch is a curved rod of almost uniform thickness, sometimes slightly pointed at the extremities, its length about half that of a tubercle-bacillus, its thickness about

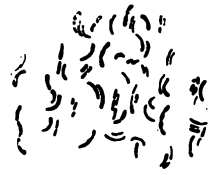


FIG. A.

From a preparation of mucus flakes of the fluid in the ileum of a case of typical cholera. Magnifying power about 700. Numbers of comma bacilli of different lengths are shown amongst numbers of small straight bacilli.

the same as that of the latter. But the comma-bacilli vary in curvature and length within considerable limits, some being just curved while others are almost semicircular, some being

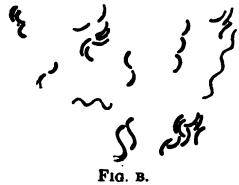


FIG. B.

From an artificial cultivation of choleraic comma-bacilli in gelatine peptone. Magnifying power 700. Most of these are single curved bacteria, a few are joined end to end in twos, thus forming S-shaped organisms; and a few are in chains of several placed end to end.

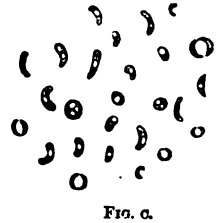


FIG. C.

From an artificial cultivation of choleraic comma-bacilli in Agar-Agar peptone at the ordinary temperature of the room after several weeks. The comma-bacilli change by vacuolation into planoconvex, then biconvex, and finally circular organisms; these by division give origin to two semicircular comma-bacilli. Magnifying power about 700.

twice and three times as long as others. They are motile, and divide transversly. In neutral Agar-Agar mixture, kept at ordinary temperature, length-divisions may be observed, see Fig. c. After transverse division they may remain joined end to end,

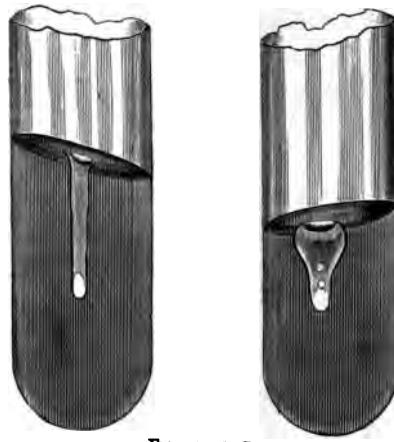
and then forming an S-shaped corpuscle. But sometimes, particularly in artificial cultivations in broth, they remain joined end to end even after repeated division, and then form either a wavy or spiral-like organism. But the type is represented by a single curved rod. For this reason it is not correct to speak of them as comma-bacilli nor as spirilla, since they correspond to what is generally considered a vibrio.

FIG. D.

Part of a test-tube containing gelatine-peptone; in it pure cultivation of choleraic comma-bacilli. The funnel-shaped opening of the channel in which the growth of the comma-bacilli is going on contains a long air bubble.

The comma-bacilli are present, amongst crowds of other putrefactive bacteria, in very varying numbers in the choleraic evacuations, sometimes very scarce, sometimes numerous; in the mucus-flakes taken from the cavity of the lower part of the ileum of typical rapidly fatal cases of cholera very soon after death they are present in small numbers, in the upper part of the ileum and in the jejunum they are either very scarce or

altogether absent. The longer the examination is delayed, of course within certain limits, the more likely are the comma-bacilli found numerously in the flakes, but not to the exclusion of other bacteria. They are generally absent from the mucous membrane itself inclusive of the epithelium of the surface loosened but not detached. No organisms of any kind are found in the tissue of the intestine, in the blood, and other tissues; putrefactive bacteria, including comma-bacilli,



FIGS. E AND F.

Pure cultivation of choleraic comma-bacilli in gelatine-peptone-broth. The two tubes had been inoculated at the same time with the same comma-bacilli, and were kept under precisely the same conditions. In both the surface of growth is marked by a depression. At the bottom of the growth is a whitish precipitate of masses of comma-bacilli. The rest of the channel is filled with almost clear liquefied gelatine.

are capable of growing after death into the clefts and spaces of the intestinal wall from the internal cavity.

The mucus-flakes of the small intestine, taken from a typical rapidly fatal case immediately after death, contain, besides detached epithelial-cells, numbers of lymph corpuscles, some perfect, others swollen up and disintegrating. Soon after death all disintegrate. These lymph-corpuscles or mucus-corpuscles contain, in varying numbers within their protoplasm, straight minute bacilli much smaller than the comma-bacilli, being only half or a fourth their length, and more or less pointed.

These small, straight bacilli are non-motile, they are never missed in a free state in the mucus-flakes, and when grown artificially they form spores. Neither the comma-bacilli nor these small bacilli show in their mode of growth, in artificial cultivations in various media, greater peculiarities than other kinds of bacteria. The peculiar mode of growth of the comma-bacilli in gelatine mixtures is shown in the accompanying woodcuts.

Both the comma-bacilli and small straight bacilli grow well in alkaline and neutral media, and are not killed by weak acids

FIG. 6.

Pure cultivation of choleraic comma-bacilli in gelatine-peptone-broth. The inoculation had been made, not in a channel, as in the previous figures, but on the surface. There is also here a depression of the growth on the surface, and in the extent of the growth the gelatine is liquefied, with a whitish precipitate at the bottom.

although they do not show growth in them, or only to a very limited degree. Neither the comma-bacilli nor the straight small bacilli can be considered as connected with the cause of cholera.

The result of the experiments performed by Nicat and Rietsch, by Koch and others, viz., death following in some of t[1] animals after injection of choleraic mucus-flakes or of cul[t] tions of comma-bacilli into the cavity of the small intestine, not due to cholera, but were probably due either to the o[1]

tion or to septicæmic poisoning. Rodents, carnivorous animals, and monkeys are altogether insusceptible to cholera. There is direct evidence that the water contaminated with choleraic evacuations only, and of course with comma-bacilli, when drunk by a large number of persons did not produce cholera; there is no definite evidence that a cholera patient elaborates cholera virus and passes it out with the evacuations.

FIG. H.

From a preparation of mucus flakes of the ileum of a case of typical cholera. Comma bacilli and minute straight bacilli, singly and in masses. Three lymph-corpuscles containing in their interior numerous small straight bacilli.

Comma-bacilli of various species have been discovered in other diseases of the alimentary canal; in the fluid of the mouth of normal persons (Lewis); in old cheese (Denike). The comma-bacilli found by Finkler and Prior in cholera nostras, differ in mode of growth from Koch's comma-bacilli of cholera. So do those found in diarrhœa due to other causes. But those of the fluid of the mouth, and those in old cheese, are identical with Koch's comma-bacilli in their mode of growth.

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